

Strategy Development for Extensive Expansion of Mesenchymal Stem Cells Isolated from Human Umbilical Cord

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Introduction: Searching for new and more easily accessible sources of human mesenchymal stem cells (hMSC), other than bone marrow, is a timely subject that is attracting more attention. Research over the last years shows that hMSC can be successfully isolated from extra-embryonic tissues discarded after birth where the frequency of MSC is substantially higher than in bone marrow, such as placenta, amnion or umbilical cord (UC). Our main objective is to establish a procedure for the production of sufficient quantities of undifferentiated hMSC for clinical applications. In this regard, we optimized culture conditions for extensive expansion of hMSC obtained from UC perivascular tissue, known as human umbilical cord perivascular cells (HUCPV-cells*) or whole UC, so called human umbilical cord matrix cells (HUCM-cells).

Materials and methods: At first 1×10^5 HUCPV-cells were seeded in culture flasks at the density of 4000 cells/cm² and grown in four slightly different α MEM (Table 1.) over 6 passages. All media were supplemented with 10% human serum (HS) and 50 μ g/ml gentamicin. In the second step 1×10^5 cells were first seeded at 7, subsequently at 3 different densities varying from 62.5 to 4000 cells/cm² (Table 2.) and propagate in α MEM containing 2 mM L-glutamine over one passage only. Cell proliferative capacity, vitality, viability and consumption of crucial nutrients (glucose, L-glutamine and essential amino acids) were monitored by microscopic (trypan blue exclusion test, colony-forming unit-fibroblast (CFU-F) test), fluorescence spectroscopic (CellTiter-Blue® Cell Viability Assay, CytoTox-One™ Homogeneous Membrane Integrity Assay, Apo-One® Homogeneous Caspase-3/7 Assay, all from Promega) and HPLC methods. The comparative analysis of cell surface epitopes (CD90, CD105, CD73, CD44, CD34, CD45 and HLA-I) were performed by using flow cytometry.

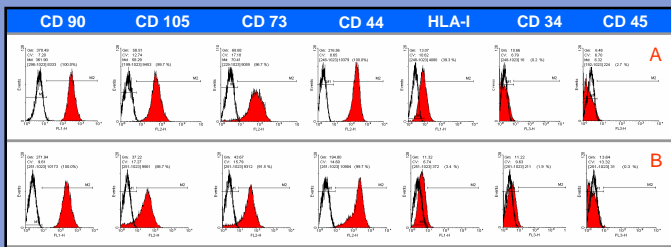


Figure 1. Immunophenotypic characteristics of HUCPV- (A) and HUCM-cells (B).

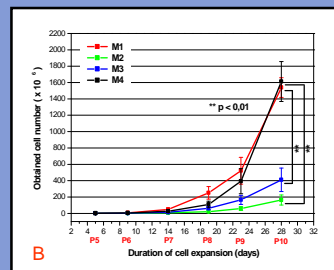
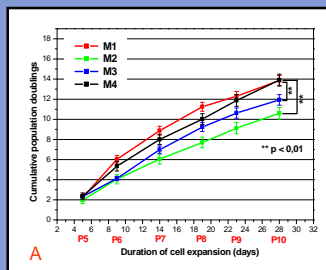


Table 1. Differences in the composition of used α MEM

Medium	Difference
M1	- Reference medium -
M2	Glutamax instead of L-glutamine
M3	Additionally buffered with HEPES
M4	Extra supplemented with osteogenic factors (0,2 mM L-ascorbate-2-phosphate, 5 mM β -glycerophosphate, 10 nm dexamethasone)

Table 2. Cell seeding densities
Total number of seeded cells: 1×10^5

Cell density (cells/cm ²)	Cell seeding area (cm ²)
4000	25
2000	50
1000	100
500	200
250	400
125	800
63	1600

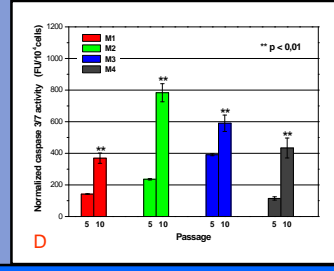
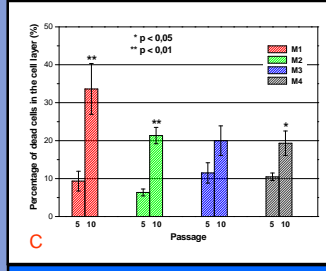


Figure 2. Cell population doublings (A), obtained cell number (B) and changes in the cell viability (C,D) during HUCPV-cell expansion over 6 passages in four different supplemented α MEM (Tab. 1).

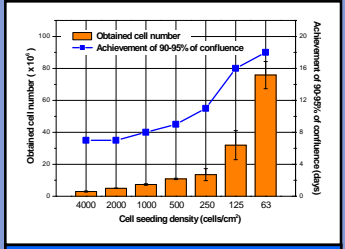


Figure 3. Relation between initial cell seeding density, number of obtained cells within one passage and time of achievement of 90-95% of confluence. In each case 1×10^5 HUCPV-cells were initially seeded.

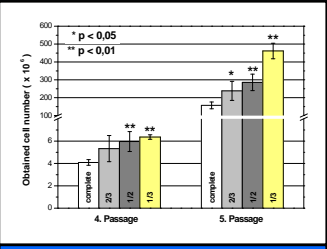


Figure 4. Influence of complete and partial (2/3, 1/2, 1/3) medium change on proliferative activity of HUCPV-cells. 1×10^5 cells were seeded at a density of 500 cells/cm² and expanded over two passages.

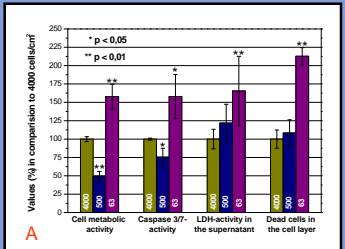
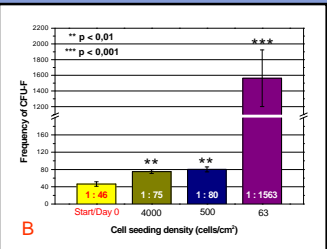


Figure 5. Influence of cell seeding density on cell viability (M) and frequency of colony-forming units-fibroblasts (CFU-F) (N). HUCM-cells were seeded at three different densities and cultivated over one passage. Parameters of cell viability were determined at the moment of ~ 90% of confluence. Parameters were first normalized to the total cell number and subsequently compared with values determined in cultures with an initial cell seeding density of 4000 cells/cm². For the CFU-F test 50 cells/cm² were seeded into 75-cm² flasks at the beginning and at the end of each cell expansion procedure. Produced colonies, comprising more than 20 cells, were counted on day 10.



Results: HUCPV- as well as HUCM-cells are highly positive for typical stem cell surface markers such as CD73, CD90, CD105 and CD44. Both exhibit remarkable proliferative activity particularly supported by α MEM containing 2 mM L-glutamine or α MEM additionally supplemented with osteogenic agents (Fig. 2. A,B). In any case cell long-term expansion is connected with deprivation of their proliferative activity and viability (Fig. 2. C,D). If initial cell seeding density of 4000 cells/cm² is used, cells must be enzymatically dissociated and splitted every 4-5 days. This process is associated with regular destruction of cell-cell and cell-matrix contacts and therefore with additional cell damage or even loss. In order to reduce the number of cell enzymatic dissociations and increase the cell number gained within one passage, we decreased the cell seeding density (Tab. 2.). Initial cell seeding at the density of 500 cells/cm² in place of 4000 cells/cm² allows to get approx. 3,5 times more cells within one passage without the intrinsic loss of cell viability (Fig. 3., 5.). In this case cell growth is additionally facilitated by partial medium change (Fig. 4.). Using this cultivation method, the routine monitoring of L-glutamine, glucose and lactate concentration in the conditioned medium and culture supernatant is mandatory. In present case regular replacement of 1/3 or 1/2 of cell culture supernatant with fresh medium was possible without limitations in the supply of crucial nutrients over two passages only (data not shown). Lower cell seeding densities than 500 cells/cm² is associated with non-uniform colonization of the cultivation area as well as with essential loss of cell viability and proliferative capacity (Fig. 5.).

Conclusions: Minor differences in the culture media supplementation, the initial density of cell seeding or the time period of cell progeny have a substantial impact on the proliferative capacity and viability of HUCPV- and HUCM-cells. Cell seeding at a density of 500 cells/cm² on a larger cultivation area allows to avoid frequent enzymatic disruption of cell-cell contacts, reduces the risk of contaminations and therefore displays a better cell expansion strategy. However further optimization of methods for the extensive expansion of these MSC, such as cell cultivation under conditions of physiological hypoxia, must be accomplished.

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